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Abstract

The aim of this study was to investigate the production of anti-*Listeria* bacteriocins by naturally occurring bacteria on silage and to characterize the most promising bacteriocin. The production of bacteriocins was evaluated by the spot-on-lawn method. The presence of interfering factors and the sensitivity to proteinase K were analyzed. The spectrum of activity was determined and the most promising bacteriocin-producing isolate was identified, being selected for the subsequent experiments. The antimicrobial peptide was extracted, analyzed regarding temperature and pH sensitivities, and purified. Of the 37 isolates evaluated, seven showed anti-*Listeria* activity. The presence of

bacteriophages and production of acidic compounds were not detected. The selected antimicrobial compound was sensitive to proteinase K and the producing-bacterium was identified as *Bacillus velezensis*. The crude extract maintained the antimicrobial activity in different temperatures and pH conditions. In conclusion, the bacteriocin produced by *Bacillus velezensis* showed strong activity against *Listeria monocytogenes*, and great stability to elevated temperature and adverse pH, desirable features for future biotechnological applications.

Keywords: Anti-*Listeria* activity; Bacteriocin; *Bacillus*.

Resumo

O objetivo deste trabalho foi investigar a produção de bacteriocinas com atividade anti-*Listeria* por bactérias naturalmente presentes na silagem e caracterizar a bacteriocina mais promissora. A produção de bacteriocinas foi avaliada pelo método de sobrecamada. A presença de fatores interferentes e a sensibilidade à proteinase K foram analisadas. O espectro de atividade foi determinado e o isolado produtor de bacteriocina mais promissor foi identificado, sendo selecionado para os experimentos subsequentes. O peptídeo antimicrobiano foi extraído, analisado quanto à sensibilidade à temperatura e ao pH, e purificado. Dos 37 isolados avaliados, sete apresentaram atividade anti-*Listeria*. Não foi detectada a presença de bacteriófagos e produção de compostos ácidos. O composto antimicrobiano selecionado foi sensível à proteinase K e a bactéria produtora foi identificada como *Bacillus velezensis*. O extrato bruto manteve a atividade antimicrobiana em diferentes temperaturas e condições de pH. Em conclusão, a bacteriocina produzida por *Bacillus velezensis* apresentou elevada atividade contra *Listeria monocytogenes*, e grande estabilidade a temperatura elevada e pH adverso, características desejáveis para futuras aplicações biotecnológicas.

Palavras-chave: Atividade anti-*Listeria*; Bacteriocina; *Bacillus*.

Resumen

El objetivo de este trabajo fue investigar la producción de bacteriocinas con actividad anti-*Listeria* por bacterias presentes de forma natural en el ensilaje y caracterizar la bacteriocina más prometedora. La producción de bacteriocinas se evaluó mediante el método de superposición. Se analizó la presencia de factores interferentes y la sensibilidad a la proteinasa K. Se determinó el espectro de actividad y se identificó el aislado productor de bacteriocina más prometedor, siendo seleccionado para experimentos posteriores. Se extrajo el péptido antimicrobiano, se analizó la sensibilidad a la temperatura y al pH y se purificó. De los 37 aislamientos evaluados, siete mostraron actividad anti-*Listeria*. No se detectó presencia de bacteriófagos ni producción de compuestos ácidos. El compuesto antimicrobiano seleccionado era sensible a la proteinasa K y la bacteria productora se identificó como *Bacillus velezensis*. El extracto crudo mantuvo su actividad antimicrobiana a diferentes temperaturas y condiciones de pH. En conclusión, la bacteriocina producida por *Bacillus velezensis* mostró alta actividad contra *Listeria monocytogenes*, y gran estabilidad a alta temperatura y pH adverso, características deseables para futuras aplicaciones biotecnológicas.

Palabras clave: Actividad anti-*Listeria*; Bacteriocina; *Bacillus*.

1. Introduction

Listeria monocytogenes is widely distributed in nature and can be found in soil, vegetation, water and intestinal tract of some animals (Reis et al., 2011). This pathogen is able to grow and adapt to different stress conditions, including a wide range of temperature (1 °C to 45 °C), acidic pH and high salt concentration (Reis et al., 2011).

The infectious disease caused by *L. monocytogenes* is called listeriosis, and eating contaminated food containing this bacterium is the main route of infection (Junior et al., 2015). Listeriosis is one of the most serious and severe foodborne diseases, affecting livestock animals, especially ruminants, but also swine, poultry and fish, as well as humans. Although the number of cases of listeriosis is not so expressive, the high rate of death associated with this infection, especially among elderly and immunocompromised patients, makes it a significant public health concern (Reis et al., 2011; Buchanan et al., 2017; Heir et al., 2018).

Regarding the animal health, the consequences of listeriosis range from a decrease in animal production and milk quality, to encephalitis, abortion and infertility (Junior et al. 2015). One of the main sources of *L. monocytogenes* for ruminants is the consumption of silage contaminated with this bacterium. Although lactic acid bacteria usually dominate silage microbiota, some pathogens, including *Listeria*, can contaminate and grow during the ensilage, a process that includes fermentation and storage of silage. In this way, the consumption of low quality silage has been associated to listeriose cases in ruminants (Durmaz et al., 2015).

As a diverse environment, with broad microbial diversity, the silage is a suitable environment for the isolation of bacteria that are able to produce antagonistic compounds (Castro et al., 2006), such as bacteriocins. Bacteriocins are ribosomally synthesized peptides, which show bactericidal or bacteriostatic activity against the target cells (Cotter et al., 2005; Drider et al., 2006; Todorov et al., 2010). Bacteriocin-producing strains have been proposed as an alternative for inhibiting spoilage microorganisms in silages, and could be used as effective inoculants to produce good quality and safe silages (Flythe and Russell, 2004; Amado et al., 2012).

The aim of this study was to investigate the production of anti-*Listeria* bacteriocins by naturally occurring bacteria on silage. The most promising bacteriocin was characterized and the producer cell was genetically identified.

2. Methodology

Microorganisms and growth conditions

Thirty seven bacteria, isolated from silage, and belonging to the culture collection of the Rumen Microbiology Laboratory, Embrapa Gado de Leite, Brazil, were randomly selected and screened for specific antagonism, in order to assess their usefulness in suppressing growth of *L. monocytogenes*. The isolates were maintained at -80 °C in glycerol containing Brain Heart Infusion (BHI) medium (10% final concentration), until use. The selected bacteria were grown in BHI medium, under aerobic conditions, at 37 °C.

Screening of antagonistic activity against indicator bacteria

Antagonistic activity was evaluated by the spot-on-lawn method (Booth et al. 1977). The silage bacteria were spotted onto BHI agar and incubated in aerobiosis, at 37 °C, for 18 h. Molten semi-solid BHI (0.8% agar), previously inoculated with *L. monocytogenes* (10^5 CFU mL⁻¹), was poured over the agar plates containing the bacterial colonies. Plates were incubated at a suitable temperature and atmosphere for each indicator organism. The antimicrobial activity was determined by the presence of inhibition zones of the indicator organism growth around the colonies of selected bacteria (>6 mm diameter).

Determination of interfering factors on the antagonistic activity

In order to verify if the antagonistic activity observed on the spot-on-lawn method was indeed due to the production of antimicrobial peptides and not to the activity of other factors, we evaluated the presence of interfering factors, such as bacteriophage presence and production of acidic compounds by the selected silage isolates.

To evaluate the presence of bacteriophage, an agar piece (3 mm) of the inhibition zone observed in the spot-on-lawn method was aseptically removed, macerated in saline (NaCl 0.9%, pH 7.0), and centrifuged (12000 g, 20 min). An aliquot of the supernatant (200 µL) was transferred to a culture medium (5 mL), previously inoculated with the indicator microorganism. After incubation period (10 min, at room temperature), an aliquot of the culture medium containing the supernatant (350 µL) was added to a semisolid BHI (3.5 mL), which was poured over a prior layer of solid BHI. After incubation in aerobiosis, at 37 °C, the plates were evaluated regarding the presence of lytic zones, which would indicate the bacteriophage presence in the selected bacteria.

To exclude the possibility of the production of acidic compounds be responsible for the antagonism, after spot-on-lawn experiment, the pH inside and outside of the growth inhibition zones were determined using pH measuring tapes.

Spectrum of action of the antagonistic substances produced by silage bacteria

The inhibitory activity of the antagonistic compounds was evaluated by the spot-on-lawn method (described above) using, as indicator microorganisms, different bacterial strains, including the culture collection strains *Escherichia coli* ATCC

25922, *Staphylococcus aureus* ATCC 25923, *Bacillus cereus* ATCC 33018, *Pseudomonas fluorescens* NCTC 10038, *Lactococcus lactis* DPC 3147, *Streptococcus salivarius* 20P3, as well as *Staphylococcus aureus*, *Streptococcus uberis*, *Streptococcus equinus*, *Pseudomonas sp.*, *Escherichia coli* and *Klebsiella sp.*, all isolated from ruminants and belonging to the culture collection of the Rumen Microbiology Laboratory.

The plates were incubated in appropriate temperature and atmosphere for each indicator microorganism, for up to 48 h. The antimicrobial activity was verified by the presence of inhibition zones larger than 6 mm in diameter.

Based on the antimicrobial activity against *L. monocytogenes* and on the activity spectrum, the most promising silage bacterium was selected for the next experiments.

Bacterial crude extracts with antimicrobial activity

Extracts of the selected silage bacteria were obtained as described by Mantovani et al. (2002), with modifications. Briefly, the pH of the stationary phase bacterial cultures was adjusted to 6.5, the cultures were heat treated (70 °C, 30 min), and the cells were harvested by centrifugation (4500 rpm, 15 min, 5 °C). The cell pellets were washed with sodium phosphate solution (5 mM, pH 6.5), centrifuged (4500 rpm, 15 min), and resuspended in acidic calcium chloride solution (100 mM, pH 2.0). After this stage, the suspension was incubated at room temperature, under stirring, for 2 h. At the end of the extraction step, the cell suspensions were centrifuged (12500 rpm, 20 min, 5 °C), and the cell free supernatant was lyophilized. The lyophilized material was resuspended in sodium phosphate solution (5 mM, pH 6.5), and named "crude extract".

The antimicrobial activity of the crude extracts was assessed by agar well-diffusion assay (Tagg et al. 1976). Molten semi-solid BHI (0.8% agar), previously inoculated with *L. monocytogenes* (10^7 CFU mL⁻¹), was poured onto sterile Petri dishes, and after solidification, 5 mm wells were drilled in the culture medium. The crude extracts were serially diluted in sodium phosphate solution (5 mM, pH 6.5), and applied (20 µL) inside the wells. The plates were kept overnight at 4 °C (for diffusion of the antimicrobial compounds from the wells), and incubated in aerobiosis, at 37 °C, for 24 h. The antimicrobial activity was expressed as arbitrary units (AU), defined as the reciprocal of the highest dilution that showed an inhibition zone of at least 9 mm in diameter (Lewus and Montville, 1991).

Proteinase K sensitivity of the crude extracts with antimicrobial activity

In order to evaluate the peptide nature of the antimicrobial compounds produced by the selected silage bacteria, a proteinase K sensitivity assay was performed. BHI-containing plates, previously inoculated with *L. monocytogenes*, were used in an agar well-diffusion assay. Proteinase K (20 mg mL⁻¹) was added (20 µL) next to each well in the culture medium; after 30 min, the crude extracts (20 µL) were applied inside the wells. The plates were kept overnight under refrigeration, and then aerobically incubated, at 37 °C, for 24 h. Non-treated crude extracts were used as a positive control for antimicrobial activity. The proteinase K sensitivity was observed by alterations of the original inhibition halos, i.e., evidence of growth of the target organism where the proteinase K was added.

Influence of temperature and pH on the biological activity of the crude extracts with antimicrobial activity

The crude extracts with antimicrobial activity obtained from the silage bacterium were submitted to different treatments to evaluate their stability under variations of temperature and pH.

In order to evaluate the thermal stability, aliquots of the crude extracts diluted in sodium phosphate solution (5 mM, pH 6.5) were heated to 45 °C, 60 °C, 80 °C and 100 °C, for 30 min. The antimicrobial activity of the crude extracts after heat treatment was evaluated by agar well-diffusion assay, using *L. monocytogenes* as indicator bacteria. Afterwards, the residual antimicrobial activity after heat treatment was compared with control samples of the crude extracts, which were kept

refrigerated. The residual antimicrobial activity was calculated according to the following equation (Cladera-Oliveira et al. 2004):

$$RA (\%) = (HT/CT) \times 100$$

Where RA is the residual antimicrobial activity, HT is the halo diameter (mm) of *L. monocytogenes* growth inhibition obtained after heat treatment of the crude extracts (measured on the agar well-diffusion assay), and CT is the halo diameter (mm) of the control samples (non-treated crude extracts).

The Britton-Robinson buffer, composed by ortho phosphoric acid (0.04 M), acetic acid (0.04 M) and boric acid (0.04 M) was used to evaluate the stability of the crude extracts at different pH values (2.0, 4.0, 8.0, 10.0 and 12.0). The pH adjustment was performed with NaOH solution (2M) and HCl (2M), and the buffers were sterilized by filtration. The crude extracts with antimicrobial activity were diluted (1:2) in the buffer with the desired pH, and the mixture was kept at room temperature, for 2 h. The antimicrobial activity of the crude extracts was then evaluated on agar well-diffusion assay. Residual antimicrobial activity was determined as described above.

Genetic identification of the selected silage bacterium

The most promising silage bacterium was identified by 16S rRNA gene sequencing. Total DNA was extracted using the phenol-chloroform method with modifications (Oliveira et al. 2002) and measured by NanoDrop (*Thermo Scientific*). The 16S rRNA gene amplification reaction was performed in DNA thermal cycler GeneAmp PCR System 9700 (*Applied Biosystems*), using the pair of universal primers 27F (AGAGTTTGATCMTGGCTCA) and 1492R (TACGGYTACCTTGTACGAC). The polymerase chain reaction (PCR) (25 μ L) was composed by 17.325 μ L milli-Q water, 2.5 μ L 10X buffer, 1.25 μ L MgCl₂ (50 mM), 0.625 μ L dNTPs (10 mM), 1 μ L primer F (10 pM μ L⁻¹), 1 μ L primer R (10 pM μ L⁻¹), 0.3 μ L Taq DNA Polymerase (5 U μ L⁻¹) (*Thermo Scientific*) and 1 μ L DNA (50 ng μ L⁻¹). The PCR conditions included an initial denaturation step at 95 °C for 5 min, followed by 35 cycles of denaturation at 95 °C for 30 s, annealing at 60 °C for 30 s, and primer extension at 72 °C for 1 min. The amplification cycle was followed by a final extension at 72 °C for 7 min, and the tubes were kept at 4 °C. The PCR products were analyzed by agarose gel electrophoresis (1.2%), stained with ethidium bromide (0.2 μ g mL⁻¹) and visualized using UV transilluminator.

The amplicons were purified using Invisorb® Fragment CleanUp kit (*Stratag Molecular*), following the manufacturer instructions. The samples were quantified with NanoDrop and the sequencing was performed by the company Myleus Facility, using the chain termination method (Sanger Method), and the ABI3730 automated sequencer (*Thermo Fisher Scientific*). All consensus sequences obtained were compared to those available in GenBank database (National Center for Biotechnology Information - NCBI), and the alignment of the sequences was performed using the Basic Local Alignment Search Tool algorithm for nucleotide (BLASTn). The sequences obtained were deposited in GenBank database.

Purification of the extract obtained from the selected silage bacterium

The lyophilized extract containing the antimicrobial peptide was purified using Fast Protein Liquid Chromatography (FPLC). The extract was resuspended in 3 mL of water/trifluoroacetic acid (TFA) 0.045% (buffer A) and applied to a reverse-phase column (MonoCap C18 Fast-Flow column 0.1 x 150 mm – GL Sciences Inc., Japan) incorporated in NanoLC - Proeminence system (Shimadzu, Kyoto, Japan) and equilibrated with 10% buffer B (acetonitrile/TFA 0.065%). After a subsequent washing with 5 min of 10% buffer B, the fraction containing bacteriocin was eluted with a linear gradient ranging from 10 to 40% acetonitrile with 0.065% TFA for 25 min; 40 to 80% for 10 min; 80% for 10 min at a flow rate of 2 μ L min⁻¹.

The absorbance was monitored at 215 nm. Eluted fractions were collected and tested regarding the anti-*Listeria* activity by the agar well-diffusion assay.

3. Results

From the 37 bacteria previously isolated from silage and selected to the evaluation of antagonist activity, seven isolates, defined as 111, 117, 120, 129, 135, 139, and 150, were capable to inhibit the growth of *L. monocytogenes*. Moreover, the presence of bacteriophages was not detected in the bacterial isolates, since no lysis plates were observed after culturing. The pH inside and outside of the growth inhibition halo was 7.0, confirming that the antagonist activity was not due the production of acidic compounds by the isolates.

Since the interference of bacteriophage presence and production of acidic metabolites on the inhibitory activity against *L. monocytogenes* were excluded, we evaluated the spectrum of activity of the antagonist compounds produced by the silage isolates. The majority of the indicator bacteria evaluated, including the Gram-negative strains, were inhibited in the spot-on-lawn test, demonstrating the broad spectrum of action of the antagonist compounds produced by the silage bacteria (Table 1).

Table 1: Spectrum of action of the antagonist compounds produced by the silage isolates, using the spot-on-lawn method.

Indicator bacteria	Silage isolates						
	111	120	129	150	135	117	139
<i>E. coli</i> ATCC 25922	-	-	-	+	-	-	-
<i>S. aureus</i> ATCC 25923	+	+	+	+	+	-	+
<i>B. cereus</i> ATCC 33018	+	+	+	-	+	-	+
<i>P. fluorescens</i> NCTC 10038	+	-	+	+	+	-	-
<i>L. lactis</i> DPC3147	-	+	-	-	+	-	-
<i>S. salivarius</i> 20P3	+	+	+	+	+	-	-
<i>S. aureus</i>	+	+	-	+	-	+	+
<i>S. uberis</i>	+	+	+	+	+	-	-
<i>S. equinus</i>	++	+++	-	+	-	-	+++
<i>Pseudomonas sp.</i>	+	+	-	+	+	-	+
<i>E. coli</i>	++	+	-	+	-	+	+
<i>Klebsiella sp.</i>	+	-	+	+	+	-	+

Antagonist activity: (-) absence of growth inhibition; presence of growth inhibition (+) inhibition zone/colony ratio between 1.04 and 1.80; (++) inhibition zone/colony ratio between 1.81 and 2.57; (+++) inhibition zone/colony ratio between 2.58 and 3.32. Source: Authors.

The antibacterial substances produced by the isolates 111 and 150 inhibited the growth of 10 indicator bacteria (83%), including the Gram-negative strains *P. fluorescens* NCTC 10038, and the environmental isolates *Pseudomonas sp.* and *Klebsiella sp.*. Other isolates, such as 117, showed a more restricted spectrum of action, inhibiting the growth of only two of the bacterial indicators evaluated. Among the indicator bacteria evaluated, *S. aureus* ATCC 25923 was the most sensitive, being inhibited by the antagonistic compounds produced by the majority of the silage bacterial strains (except the isolate 117). On the other hand, *E. coli* ATCC 25922 was inhibited only by the antagonistic compound produced by the isolate 150.

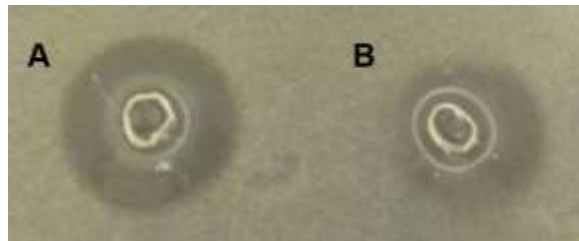
Based on the anti-*Listeria* activity and on the broader spectrum of action, the isolate 150 was selected for the subsequent experiments. The crude extract obtained from the isolate 150 showed strong anti-*Listeria* activity, and a zone of *Listeria* growth inhibition measuring 21 mm of diameter was observed in the agar well-diffusion assay (Figure 1). The crude extract obtained from the isolate 150 was sensitive to proteinase K, losing its antagonist activity against *L. monocytogenes* after the treatment with the proteolytic enzyme. This effect was observed after changing in the form of the inhibition zone in the agar well-diffusion assay (Figure 2).

Figure 1: Inhibition activity of the crude extract obtained from the isolate 150 against *Listeria monocytogenes*. The agar well-diffusion assay was used.



Source: Authors.

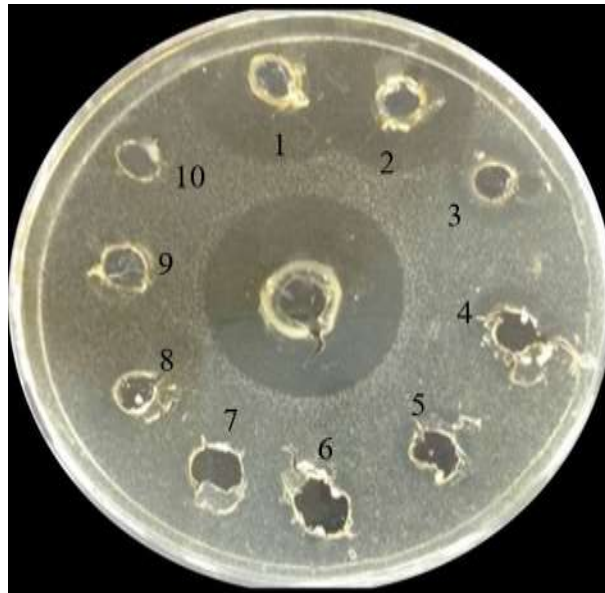
Figure 2: Sensitivity to proteinase K of the crude extract with antimicrobial activity obtained from the isolate 150. Inhibition zones around the agar well are shown. **A)** Crude extract activity; **B)** Crude extract activity after proteinase K treatment. *Listeria monocytogenes* was used as indicator microorganisms in agar well-diffusion assay.



Source: Authors.

Once the peptide character has been confirmed by the protease K sensitivity, the antibacterial activity of the crude extract was determined in arbitrary units (AU mL⁻¹), being obtained an activity of 1600 AU mL⁻¹ against *L. monocytogenes* (presence of *Listeria*-growth inhibition zones until the dilution 1:32) (Figure 3).

Figure 3: Agar well-diffusion assay showing the inhibitory activity of the crude extract obtained from the isolate 150, using *Listeria monocytogenes* as indicator bacterium. From the crude extract, a serial dilution was prepared with twice increment (1:2 until 1:1024). Central well: crude extract; well 1: crude extract diluted 1:2; well 10: dilution 1:1024.

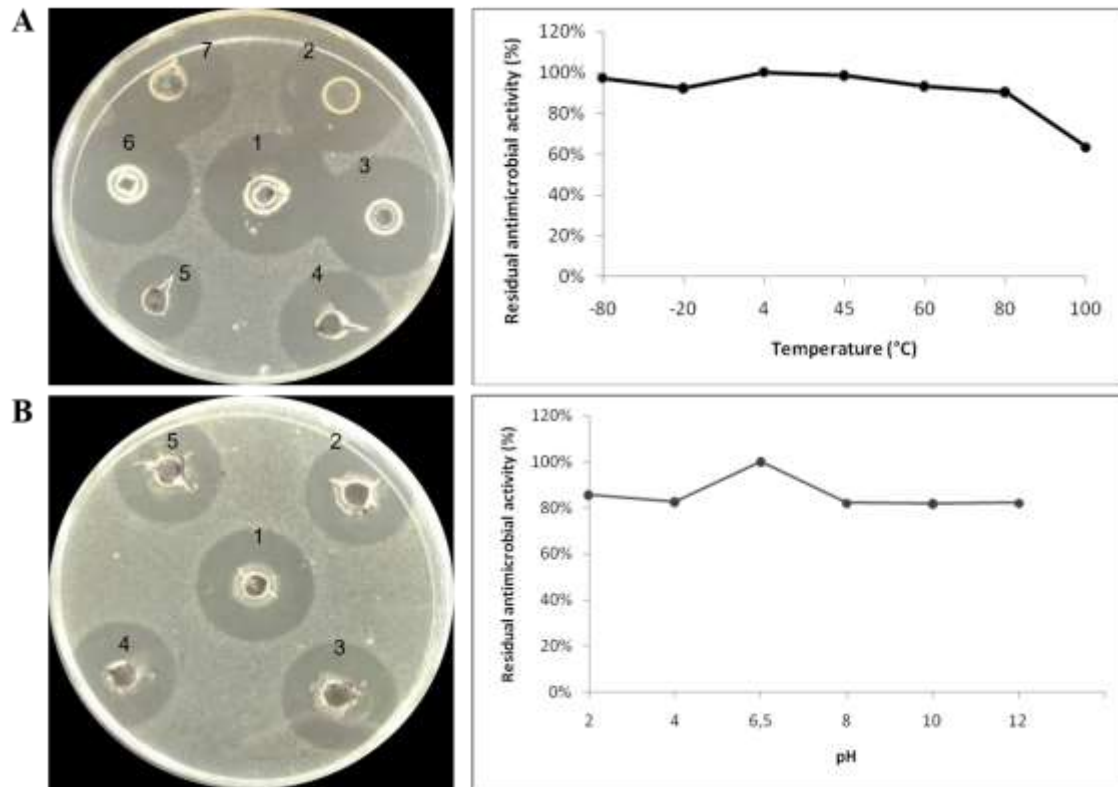


Source: Authors.

The crude extract obtained from the isolate 150 showed good stability in different temperatures, maintaining its anti-*Listeria* activity after heating and freezing (Fig. 4A). Considering the crude extract stored at 4 °C as a control (100% of anti-*Listeria* activity), when the extract was submitted to 45 °C, the residual activity (RA) observed was 98%; a RA of 93% was detected at 60 °C, and at 80 °C the RA was 90%. A bigger reduction in the anti-*Listeria* activity was observed when the crude extract was submitted to 100 °C (RA = 64%). The crude extract was also stable at lower temperatures, showing a RA of 92% and 97% when stored at -20 °C and -80 °C, respectively (Figure 4A).

Considering the pH 6.5 as control (original pH of the extract), the growth inhibition zone for *L. monocytogenes* was determined as 28.4 mm (100% antimicrobial activity). The crude extract showed a considerable stability in different pHs (Figure 4B). In all the pHs tested, the biological activity of the crude extract was more pronounced at pH 2 (RA > 85%), and the residual activity remained over than 80% in the other pH values (4-12) (Figure 4B).

Figure 4: Influence of the temperature and pH on the anti-*Listeria* activity of the crude extract obtained from the isolate 150, evaluated by the agar well-diffusion assay and graphed according to the residual antimicrobial activity. **A)** Thermal stability: (1) 4 °C; (2) 45 °C; (3) 60 °C; (4) 80 °C; (5) 100 °C; (6) -20 °C; (7) -80 °C. **B)** pH stability: (1) pH 2; (2) pH 4; (3) pH 8; (4) pH 10; (5) pH 12.

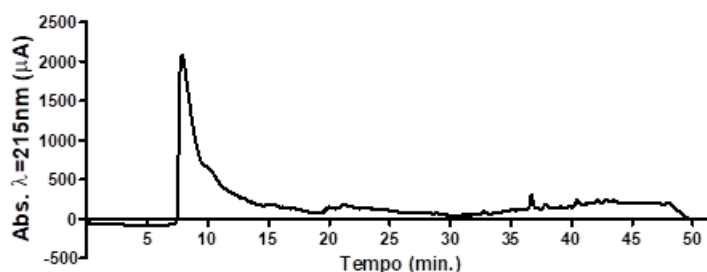


Source: Authors.

Analyses of similarity among the 16S rRNA gene sequence showed that the silage isolate evaluated showed high identity to *Bacillus velezensis* strain FZB42 (NR075005.2). The 16S rRNA gene sequence of the silage isolate (isolate 150) was deposited in the GenBank sequence database under accession number MK658866.

The crude extract obtained from *B. velezensis* (isolate 150) was purified in FPLC, using C18 column (Figure 5). The fractions were collected and the predominant peak, which appeared in the chromatogram after 7 minutes of purification, exhibited strong anti-*Listeria* activity, showing a halo of growth inhibition of 18 mm diameter in the agar well-diffusion assay (the crude extract before purification showed a zone of *Listeria* growth inhibition measuring 21 mm of diameter). The minor peaks observed in the chromatogram did not show antimicrobial activity.

Figure 5: A fast protein liquid chromatogram of the crude extract obtained from *Bacillus velezensis* (isolate 150). The extract obtained from cells by acidic CaCl_2 (100 mM, pH 2) was collected from a reverse-phase column (MonoCap C18 Fast-Flow column 0.1 x 150 mm) using trifluoroacetic acid 0.065% and a linear gradient of acetonitrile. The major peak obtained was tested and shows antimicrobial activity.



Source: Authors.

4. Discussion

Listeriosis can cause severe sequels to animal health. The clinical form of the disease can be characterized in three ways: septicemia, with abscesses in viscera, such as liver and spleen, abortion and meningoencephalitis (Campero et al., 2002). The acquisition of *L. monocytogenes* by animals can occur by the ingestion of water contaminated with feces or secretions of infected animals (Brugere-Picoux 2008), and, to ruminants, the main sources of this microorganism is soil and contaminated food (especially poorly fermented silage) (Fentahum and Freseberat, 2012).

Lactic acid bacteria (LAB) have been extensively used in order to inhibit the growth of undesirable microorganisms (Dolenčić Špehar et al., 2020). In addition to microbial control, LAB can also be used to improve the fermentation process in silage, contributing for silage quality (Cheng et al., 2021). Many of LAB are able to produce antimicrobial compounds, such as bacteriocins, that can control the bacterial community during the ensilage process (Contreras-Govea et al., 2011). The addition of *Lactobacillus buchneri*, a producer of the bacteriocin buchnericin LB, on sugarcane silage, for example, has been reported to inhibit the growth of undesirable bacteria, such as *Listeria sp.*, *Bacillus sp.*, *Micrococcus sp.*, *Enterococcus sp.*, *Streptococcus sp.*, *Lactobacillus sp.*, *Leuconostoc sp.* and *Pediococcus sp.*, improving the quality of the silage and reducing the loss of dry matter during the ensilage process (Yildirim 2001; Zopollatto et al., 2009). However, these bacterial inoculants might not prevent secondary fermentation in moist silages, sometimes impairing the aerobic stability of grass and small grain silages (Weinberg and Muck, 1996). Therefore, it is important to evaluate other microorganisms than LAB as alternative inoculants to silage.

In the present study, 18.9% of the bacterial strains isolated from silage showed antibacterial activity, inhibiting the growth of *L. monocytogenes*, and other Gram-positive bacteria, including the pathogenic strains *S. aureus* and *B. cereus*, as well as Gram-negative bacteria, such as *E. coli*, *Pseudomonas* and *Klebsiella*. The activity spectrum of bacteriocins is generally associated to phylogenetically-related bacteria (Bordignon-Junior et al. 2013; Yang et al. 2014), although, as also demonstrated in the present study, some bacteriocins have shown a broad spectrum of action (Ivanova et al., 1998; Todorov et al., 2010; De Oliveira et al., 2012). It is important to highlight the activity of the bacteriocins produced by the silage isolates evaluated against *S. aureus* and *E. coli*, important pathogens that cause bovine mastitis, a disease still prevalent in the Brazilian herd that leads to considerable economic losses (Fagundes and Oliveira, 2004).

The most promising bacteriocin-producing bacterium evaluated in the present study was identified as *B. velezensis*. The genus *Bacillus* is a heterogeneous group of thermophilic Gram-positive endospore-forming bacteria, widespread in the environment, being the soil its main reservoir (Galvez et al., 1993). The production of bacteriocins have been reported in

different strains of *Bacillus*, including cerein produced by *B. cereus* (Oscariz et al., 1999; Oscariz et al., 2006), bacillocin 490 by *B. licheniformis* (Martirani et al., 2002), thuricin by *B. thuringiensis* (Gray et al., 2006), haloduracin by *B. halodurans* (Lawton et al., 2007), and subtilosin by *B. amyloliquefaciens* (Sutyak et al., 2008). The peptides produced by *Bacillus* strains usually show a broader spectrum of activity (Abrioel et al., 2011; Ansari et al., 2012). *B. velezensis* strain LS69, isolated from rice field, showed antagonist activity against several pathogens, including *L. monocytogenes*, and its genome sequencing reveals biosynthesis genes for the bacteriocins amylolysin and amylocyclin (Liu et al., 2017). Another strain of *B. velezensis* (GF610), isolated from soil, produces the amyloliquecidin, with potent anti-*Listeria* activity (Gerst et al., 2017).

In this study, the protein character of the antibacterial compound produced by *B. velezensis* was confirmed by the proteinase K sensibility. The bacteriocin was stable to different wide of temperature and pH, as expected for a bacterium capable of withstanding extreme environmental conditions (Abrioel et al., 2011; Baruzzi et al. 2011; Ansari et al. 2012). The exposure of *B. velezensis* bacteriocin to low or high temperatures resulted in a slight reduction in its anti-*Listeria* activity. At temperatures below 80 °C, the residual anti-*Listeria* activity remained above 90%, while at 100 °C this activity decreased to 64%. Lim et al., (2016) demonstrated similar results, being the residual activity of 100% when the bacteriocin produced by *Bacillus amyloliquefaciens* was exposed to 4 °C, 37 °C, 50 °C and 80 °C for 30 min, while in 100 °C this activity decreased to 80%.

Even showing higher antimicrobial activity at neutral pH (pH = 6.5), the bacteriocin produced by *B. velezensis* showed considerable stability in different pH values, maintaining over than 80% of its anti-*Listeria* activity in the pH range of 2 to 12. Similarly, stability to pH levels ranging from 3.0 to 9.0 was reported for mejucin, produced by *Bacillus subtilis* SN7 (Lee and Chang, 2018). On the other hand, a pH-dependent antimicrobial activity has been demonstrated for other bacteriocins, such as nisin, lactoestrepicin (Bromberg et al. 2006), and bovicin HC5 (Paiva et al., 2011). The stability of the bacteriocin prospected in our study to different pH and temperature value is important to its potential application.

5. Final Considerations

From the literature survey, it has been found that *B. velezensis*, a growing fast bacterium, is harmless to human and animal, and does not pollute the environment, playing an important role as a probiotic in animal feed. These properties, associated with the production of a broad inhibitory spectrum, pH-tolerant and thermo-stable bacteriocin, characterized in our study, supports and suggests the use of *B. velezensis* and its bacteriocin as inoculants for silage, alone or in combination with other methods, specially focusing on *L. monocytogenes* proliferation control.

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